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| (54) Title: METHOD FOR THE PROTECTION OF PLANTS AGAINST PATHOGENS | | | |
| (57) Abstract A method for the protection of plants against pathogens, wherein a polynucleotide sequence comprising at least a sequence of a pathogenic avirulence gene (E) encoding a specific elicitor protein molecule (e) or a portion thereof is introduced into the genome of a plant containing a corresponding resistance gene (R), in which genes (E) and (R) are regulated in such a manner that simultaneous expression of said genes only occurs at the site of infection and said simultaneous expression can be induced by a broad range of pathogens. A polynucleotide sequence comprising at least a sequence of an avirulence gene (E) from a plant pathogen encoding a specific elicitor protein molecule (e) or a portion thereof, and a plant promoter (P) that can be induced by a broad range of pathogens and which permits expression at the site of infection only. Plant obtainable by use of said method and plant comprising said polynucleotide sequence. | | | |

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Method for the protection of plants against pathogens

The invention is directed at a method for the protection of plants against pathogens, also at a polynucleotide sequence comprising an avirulence gene (E) from a pathogen regulated by a pathogen inducible promoter, and at a polynucleotide sequence comprising a corresponding resistance gene (R) regulated by a pathogen inducible promoter, whereby said DNA-sequences can be used in said method. The invention is also directed at a plant obtained via said method.

During evolution many fungi and bacteria that are pathogenic for plants have specialised in only one host species or sometimes even in one variety of said species. Thus pathogenic races can be found that colonise only certain cultivars of the host and do not colonise other cultivars. In the latter case the cultivars are resistant by means of a quick inducible defence mechanism.

In order to carry out a successful colonisation of the plant the pathogen must evade, suppress or nullify the existing defence mechanism of the plant. In genetic terms the specific race cultivar interactions can be described by a gene-for-gene model, whereby a protein elicitor molecule (e) encoded by a pathogen avirulence gene (E) interacts with a receptor protein molecule (r) encoded by a plant resistance gene (R) and thereby induces the defence mechanism, which often becomes phenotypically visible as the hypersensitive response (HR): the local death of a few plant cells, which simultaneously destroys the pathogen (De Wit, 1986).

The genetics of gene-for-gene interactions have been well described in the literature, especially the interactions between pathogenic fungi and plants (I.R. Crute, 1985), yet very little is known about the biochemical and molecular mechanisms (De Wit, 1987, Collinge and Slusarenko, 1987).

A pathogen that does not possess the avirulence gene or a pathogen in which the avirulence gene is not expressed does not trigger the host defence system and subsequently a successful colonisation can occur: in this case the host plant is susceptible.

Various race specific avirulence genes have been cloned from various plant pathogenic bacteria, whereby virulent races have been transformed with genomic clones of avirulent races and were subsequently tested for avirulence on plant genotypes with the corresponding resistance gene (Staskawicz et al., '84, Staskawicz et al. '87, Shintaku M.W. et al., '89, Vivian et al. '80, Hitchin et al. '89). This method of isolation of bacterial avirulence genes is however not applicable for fungal avirulence genes due to the low transformation efficiency and the lack of suitable cloning systems, such as cosmid vectors with a broad host range. The only fungus for which an efficient transformation system with an autonomously replicating vector has been determined is Ustilago maydis (Leong, 1989).

It has been found that plants can be offered a broad protection against pathogens by introducing a polynucleotide sequence comprising at least a sequence of a pathogen avirulence gene (E) encoding a specific elicitor protein molecule (e) or a portion thereof, into the genome of a plant containing a corresponding resistance gene (R); and providing means for regulating the expression of said genes in such a manner that simultaneous expression occurs only at the site of infection and induction of said simultaneous expression can be achieved by a broad range of pathogens.

It is possible to introduce the above-mentioned polynucleotide sequence comprising at least a sequence of a pathogen avirulence gene (E) into a plant that contains resistance gene (R) that is at least expressed at the site of infection, putatively even constitutively in the whole plant. In this case the avirulence gene (E) must be regulated by a promoter that is induced by a pathogen and only permits expression at the site of infection, in order to avoid the induction of the hypersensitive response in the whole plant.

The hypersensitive response is only permitted to be activated by a pathogen or an aspecific elicitor produced by a pathogen. The hypersensitive response must not or hardly be inducible by other exterior stimuli and should be restricted to an area surrounding the site of infection. Without these restrictions

the activation would result in the virtual destruction of the plant.

It is also possible to introduce a resistance gene (R) corresponding to the avirulence gene (E) into a plant that does not already contain the corresponding resistance gene (R). This can be achieved via breeding or via genetic manipulation techniques.

In the latter case it is possible to introduce a polynucleotide sequence comprising at least a sequence of a resistance gene (R) or a portion thereof, and a plant promoter (P) that can be induced by a broad range of pathogens and in such a manner that the product of resistance gene (R) is only expressed at the site of the infection. In this instance it is even possible for the avirulence gene (E) to be constitutively expressed even in the whole plant. It is also possible for gene (E) and gene (R) to be regulated by identical promoters provided they are strictly inducible at the site of the infection and only by a pathogen.

In the above mentioned embodiments the pathogen-inducible promoter to be employed is required to:

- a) be induced by all or most of the plant's pathogens or aspecific elicitors produced by said pathogens;
- b) be virtually only inducible by pathogens and not or hardly inducible by other exterior stimuli;
- c) be only able to express the genes that are controlled by the promoter very locally and never systemically.

In another embodiment of the invention gene (E) or (R) can be tissue specific and the other gene must be pathogen inducible at the site of the infection only in tissue for which the first gene is tissue specific. In this instance it is for example possible to introduce a gene (R) that is expressed only in the roots of the plant and a gene (E) that is induced locally by a pathogen in the roots yet constitutively in other tissues, whereby protection against pathogens is obtained for the roots.

The avirulence gene (E) can be derived from a fungus, a bacterium, a virus or a nematode. An example of a plant pathogenic fungus from which an avirulence gene can be readily derived is Cladosporium fulvum.

The resistance gene (R) can be used that is naturally

present f xample in a plant that is a ber of tne family or the Solanaceae, such as a plant of the species Lycopersicon esculentum. It is possible to introduce this gene (R) into another member of the family of the Solanaceae by breeding. Said other
5 member can either already contain the stringently regulated avirulence gene (E) or else said avirulence gene can be subsequently introduced into the plant via genetic manipulation techniques. The resistance gene (R) can obviously also be introduced via genetic manipulation techniques.

10 The choice of the combination of avirulence gene (E) and resistance gene (R) as well as the promoters to be introduced into the plant, be it through breeding or genetic manipulation techniques, will depend on the plant variety that is to be protected and whether resistance gene (R) is naturally present in
15 the plant.

In the whole plant kingdom pathogen inducible plant promoters are known that are induced by a broad range of pathogens and by aspecific elicitors produced by these pathogens. Such plant promoters are also known that are only expressed very locally and
20 never systemically. Matton and Brisson (1989), for example describe the nucleotide sequence of a cDNA clone (pSTH-2) corresponding to mRNA sequences that specifically accumulate in potato after elicitation with nonspecific elicitors of P. infestans (Marineau et al., 1987), as well as a closely related clone, pSTH-21, that shows
25 great similarity in amino acid sequence with the cDNA-clones corresponding to the elicitor and pathogen induced pathogenesis related proteins from the pea (42%) (Fritensky et al., 1988) and parsley (37%) (Somsisch 1988). Matton and Brisson (1989) also describe the accumulation of mRNA's corresponding to said clones
30 pSTH-2 and pSTH-21 in various potato tissues and in tomato leaves. Somsisch (1986) describes how the de novo synthesis of pathogenesis related (PR) proteins in cultured parsley cells can be achieved by treatment with fungal elicitor. In this system PR-protein synthesis is preceded by mRNA synthesis resulting from fast
35 and temporary activation of the corresponding genes. Such activation is also observed with intact parsley plants upon fungal infection (Somsisch, 1988) and is accompanied by massive yet local

accumulation of RNA around the infection source. In general genes involved in the synthesis of phytoalexins are induced very locally in plants by various types of pathogens and their specific elicitors (Hahlbrock, H. and Scheel, D., 1989; Kuc, J. and Rush, J.S., 1985).

One of the methods that can be used for the detection of a resistance gene (R) from a plant, whereby the product (r) of said gene shows interaction with a specific elicitor protein molecule (e) that is encoded by a pathogen avirulence gene (E) can be described as follows; in this method a specific elicitor protein (e), a resistance gene product (r) and subsequently a resistance gene (R) are isolated with the aid of the product of an avirulence gene (E). In principle this method can be used for the isolation of any resistance gene product (r) and the encoding resistance gene (R) when the corresponding avirulence gene (E) and its product (e) are known. The method can be represented as follows; a cDNA-library of a plant containing the resistance gene (R) is made in an expression vector, whereby the product of (R), the receptor (r) is produced. A positive clone is detected in the cDNA-library by binding a specific elicitor protein molecule (e) to the receptor protein (r). The binding (complexing of (e) to (r)) is made visible by providing (e) with a detectable label. This positive cDNA-clone contains the coding sequence for the resistance gene. The intact gene (R) can be detected with this cDNA-clone from the genomic library from the plant containing the resistance gene. With the aid of the cDNA or genomic clone a plant lacking the resistance gene can be transformed; positive transformants are screened for possession of the resistance gene by inoculation (contamination) with the appropriate pathogen. The cloned gene (R) can be introduced into plants via either genetic manipulation techniques or breeding.

Alternatively the resistance gene (R) can be cloned by published methods of transposon tagging, chromosome walking and genomic substitution (Dickinson, M.D. et al, 1991).

It is highly unlikely that pathogens will develop tolerance to a plant comprising the two components, i.e. gene (E) and gene (R) (the two component sensor system), obtained via the method

according to the invention, due to the use of aspecific inducible plant promoters, inducible by all pathogens, to induce the simultaneous local expression of the avirulence gene (E) and the resistance gene (R). It is necessary for at least one of the promoters regulating either the avirulence gene (E) or the resistance gene (R) to be only inducible at the site of the infection, in order to avoid destruction of virtually the whole plant, which is obviously undesirable.

As the two component sensor system according to the invention can be obtained with every combination of avirulence gene-resistance gene in any plant in which the avirulence gene and the resistance gene can be expressed, it is very broadly applicable against many if not all pathogens for the plant kingdom.

The two component sensor system provides an excellent solution to reduce the use of the pesticides now frequently used against pathogens. In time this will make it possible to relieve the environment of a large part of these agents.

By way of example, a description of the preparation of a two component sensor system according to the method of the invention is given.

EXAMPLE

An avirulence gene of Cladosporium fulvum (namely the avr9 avirulence gene, that was previously referred to in the literature as A9) is applied as avirulence gene in combination with the corresponding resistance gene Cf9, that is naturally present in a tomato cultivar. This two component sensor system can at least be propagated by breeding into varieties of the genus Lycopersicon and a part of the Solanaceae family and can optionally be introduced via genetic manipulation techniques in said plants or in other families. Various fungally encoded race specific elicitor molecules have been identified that induce necrosis on tomato cultivars containing the corresponding resistance genes (De Wit and Spikman 1982; De Wit et al. 1985). Such a race specific elicitor molecule, the product of avirulence gene avr9, has been purified to homogeneity. The purified protein induced fast and local necrosis

upon injection into leaves of tomato genotypes containing the resistance gene Cf9. In genotypes containing other Cf-genes this did not occur. The amino acid sequence of the purified elicitor molecule was determined (Schottens-Toma and De Wit 1988). The elicitor molecule was formed in all compatible interactions between tomato-C. fulvum involving races of fungi that were avirulent on tomatoes Cf9 genotypes, however at no single interaction involving races of fungi that are virulent on Cf9 genotypes (Scholtens-Toma et al. 1989). In order to detect the mRNA encoding the necrosis inducing protein, i.e. an elicitor protein, 4 oligonucleotide probes were synthesised derived from the amino acid sequence (Fig. 1). The oligonucleotides contain either mixtures of nucleotides (such as in probe B) or inosines (such as in probe D) or a combination of both (such as in probes A and C). All four oligonucleotides were labelled at the 5'-terminus and hybridised to identical Northern blots containing identical amounts of poly(A)-RNA derived from healthy tomato plants, in vitro cultured C.fulvum and 3 different compatible tomato-C.fulvum interactions. Fig. 2 shows that probe B specifically hybridised to a mRNA of approximately 600 nucleotides that was present in two compatible interactions namely: cultivar Cf4/race 4 (lane 3) and cultivar Cf5-race 5 (lane 4). This mRNA was not found in tomato plants that were not infected (lane 1) or in C. fulvum cultivated in vitro (lane 2). Neither was any hybridisation observed in the interaction of cultivar Cf5 with race 2.4.5.9.11 (lane 5), as could be expected for an interaction of a race that is virulent on tomato Cf9 genotypes. Thus it was concluded that probe B detected mRNA for the necrosis inducing protein. Probes A, C and D did not detect specific mRNA's as is shown in Fig. 2.

Oligonucleotide probe B was used in a primer-extension experiment. The oligonucleotide was labelled at the 5'-terminus and hybridised to equal amounts of poly(A)-RNA derived from compatible interactions of cultivar Cf5 with either race 5 or race 2.4.5.9.11 (represented respectively in Fig. 1, lanes 4 and 5). The primer was extended with reverse transcriptase and the extension products were analysed on a PAGE-gel. Fig. 3 shows that a specific extension product was formed on poly(A)-RNA derived from

the interaction of cultivar Cf5/race 5 (lane 1), however not on poly(A)-RNA derived from the interaction of cultivar Cf5/race 2.4.5.9.11 (lane 2). The size of the extension product was approximately 270 nucleotides which indicated that avr9 mRNA possesses approximately 200 nucleotides for the sequence encoding the necrosis inducing protein.

Poly(A)-RNA derived from the interaction of Cf5/race 5 (represented in Fig. 2, lane 4) was used to prepare a cDNA library in lambda gt11. A library was obtained containing 100.000 independent recombinants. Examination of filters containing 5000 phages with terminally labelled oligonucleotide probe B, resulted in the isolation of two possible candidates, one that hybridised weakly (phage A9-1), and one that hybridised appreciably better (phage A9-2). Both phages were purified and the DNA was isolated. The phage DNA was labelled and hybridised to blots that were identical to the blot shown in Fig. 2. Phage A9-1 hybridised to an mRNA containing approximately 1500 nucleotides and was present in a small amount in the three interactions between tomato and *C.fulvum*. This phage did not contain cDNA corresponding to the mRNA observed in Fig. 2 and was not analysed further.

The labelled DNA of phage A9-2 hybridised with an mRNA of approximately 600 nucleotides that was only present in the compatible interactions of cultivar Cf4/race 4 and cultivar Cf5/race 5 i.e. in a pattern corresponding to the hybridisation observed with oligonucleotide probe B. Therefore phage A9-2 contained a copy of the mRNA encoding the necrosis inducing protein. The cDNA present in phage A9-2 was subcloned and the sequence was determined. The insertion had a length of 405 base pairs and corresponded to the 3'-terminus of the mRNA including a poly(A)-tail of 20 nucleotides. The insertion encoded the whole sequence of the necrosis inducing protein and was contained within a longer open reading frame. It was estimated from the position of the oligonucleotide probe B in the DNA sequence and the size of the primer extension product that the insertion of clone A9-2 lacked approximately 110 base pairs at the 5'-terminus of the mRNA. In order to obtain a full length cDNA-clone the cDNA-library was examined again with a labelled RNA-probe containing 70 nucleotides

of the 5'-terminus of the insertion of clone A9-2. Three different phages A9-3, A9-5 and A9-8 were obtained and their insertions were subcloned and sequenced. The sequence of the three clones was completely identical to the sequence of clone A9-2 in the overlapping regions. The 4 clones contain poly(A)-tails commencing at different positions in the sequence. It was derived from the primer extension experiment shown in Fig. 3 that the largest clone (A9-3) lacked approximately 35 nucleotides. Therefore a new primer was designed that hybridised at position 75-100. This primer was used in a primer extension experiment on poly(A)-RNA in the presence of dideoxynucleotides. This RNA-sequence led to the addition of another 24 nucleotides in front of the insertion of A9-3. Other final products were observed that were 5-20 nucleotides longer than the major extension product. The various final products of the primer extension were not caused by degradation of mRNA, as one extension experiment with a primer for a different mRNA provided only one discrete extension product with the correct size. The sequence of the avr9 cDNA and the structure of the corresponding cDNA-clones is shown in Fig. 4. The isolation and characterisation of the cDNA-clones revealed that the necrosis inducing protein is formed as a precursor protein of at least 63 amino acids. Surprisingly the DNA sequence revealed an additional histidine codon at the C-terminus of the sequence of the mature elicitor molecule. It has previously been described that the elicitor molecule had a length of 27 amino acids (Scholtens-Toma and De Wit, 1988). Re-examination of the protein sequence data, however, confirmed the presence of an additional histidine residue at position 28. This residue had been overlooked during the original analysis of the protein sequence due to a low signal obtained with this amino acid. The molecular weight of the mature avr9 elicitor protein molecule is 3189 Dalton.

A genomic clone of the avirulence gene avr9 has been isolated from a genomic library (in vector lambdaEMBL3) of race 5 of C. fulvum using the cDNA clone A9-2. Sequence analysis revealed a 59 base pair intron, a putative TATA-box and several repeats in the promoter and terminator region. Stable transformants of race 2.4.5.9.11 were obtained with the genomic clone after co-

transformation with pAN7 (hygromycine resistance). The wild type race 2.4.5.9.11 is virulent on Cf9 genotypes of tomato but the transformant was not. This means that cultivar specificity of these transformants was converted phenotypically from virulent to avirulent on Cf9 genotypes, indicating that the avirulence gene *avr9* has changed the genotype of race 2.4.5.9.11 into that of race 2.4.5.11. This experiment proves that avirulence gene *avr9* is indeed the causal inducer of resistance in combination with the Cf9 resistance gene. Hence, it can be concluded that locally simultaneous expression of an avirulence gene (E) and a corresponding resistance gene (R) in a plant gives rise to local necrosis.

References

1. Collinge, D.B. and Slusarenko, A.J. 1987, Plant gene expression in response to pathogens., *Plant.Mol.Biol.* 9, 389-410
- 5 2. Crute I.R. (1985). The genetic basis of relationships between microbial parasites and their hosts. "Mechanisms of resistance to plant diseases", (Ed. Fraser R.S.S.), 80-142, M.Nijhoff/Dr.W.Junk Uitg.Mij., Dordrecht
3. De Wit P.J.G.M. and Spikman G. (1932), *Physiol.Plant Pathol.* 21, 1-11
- 10 4. Dickinson, M., Jones, D., Thomas, C., Harrison, K., Englisch, J., Bishop, G., Scofield, S., Hammond-Kosack and Jones, J.D.G. (1991) Strategies for the cloning of genes in tomato for resistance to Fulvia fulva. In: advances in Molecular Genetics of Plant-Microbe Interactions; Volume 1. (eds. Hennecke, H., Verma, D.S.) Kluwer Academic Publishers, Dordrecht, Boston, London.
- 15 5. Fristensky B., Horovitz D., and Hadwiger L.A. (1988). cDNA Sequences for pea disease resistance response genes. *Plant Molecular Biology* 11, 713-715
- 20 6. Hahlbrock, H. and Scheel, D., (1989) Physiology and molecular biology of phenylpropanoid metabolism. *Annual Review of Plant Physiology and Plant Molecular Biology* 40, p 347-369
7. Hitchin F.E. et al. (1989) *Physiol. Molec. Plant Pathol.* 35, 335-344.
- 25 8. Kuc, J. and Rush, J.S., (1985), Phytoalexins. *Archives of Biochemistry and Biophysics* 236, p455-472
9. Leong S.A. and Holden (1989), *Annu.Rev.Phytopathol.* 27, 463-481.
- 30 10. Marineau, C., Matton D.P., and Brisson, N. 1987. Differential Accumulation of potato tuber mRNA's during the hypersensitive response induced by arachidonic acid elicitor. *Plant Mol.Biol.* 9, 335-342.
- 35 11. Matton D.P. en Brisson N. (1989). Cloning, expression, and sequence conservation of pathogenesis-related gene transcripts of potato. *Molecular Plant-Microbe Interactions* 2, 325-331.

12. Scholtens-Toma I.M.J., De Wit G.J.M. en De Wit P.J.G.M. (1989) *Neth.J. Plant Pathol.* 95, suppl.1, 161-168.
13. Scholtens-Toma I.M.J. and De Wit P.J.G.M. (1988) *Physiol.Mol c. Plant Pathol.* 33, 59-67
- 5 14. Shintaku M.H. et al. (1989) *Physiol. Molec.Plant Pathol.* 35, 313-322.
15. Somssisch I.E., Schmelzer E., Bollmann J. and Hahlbrock K. 1986. Rapid activation by fungal elicitor of genes encoding "pathogenesis-related" proteins in cultured parsley cells. *Proc.Natl. Acad.Sci. USA* 83, 2427-2430.
- 10 16. Somssich I.E., Schmelzer E., Krawinkel P. en K. Hahlbrock (1988). Gene structure and in situ transcript localization of pathogenesis-related protein 1 in parsley. *Molecular and General Genetics* 213, 93-98.
- 15 17. Staskawicz B.J., Dahlbeck D., Keen N.T. (1984) *Proc.Natl.Acad.Sci. USA* 81, 6024-6028.
18. Staskawicz B. et al. (1987) *J. Bacter.* 169, 5789-5794.
19. Vivian A. et al. (1989) *Physiol. Molec. Plant Pathol.* 35, 335-344.
- 20 20. de Wit P.J.G.M. and Hofman J.E. and Velthuis G.C.M. and Kuc J.A. (1985) *Plant fysiolo.* vol. 77, pp 642-647. Isolation and characterisation of an elicitor of necrosis isolated from intercellular fluids of compatible interactions of *Clad. fulvum* (syn. *fulvia*, *fulva*) and tomato.
- 25 21. de Wit P.J.G.M. -Specificity of active resistance mechanisms in plant-fungus interactions in: "Fungal infection of plants ed. G.F. Pegg and P.G. Ayres" pp 1-24, Cambridge University Press.
- 30 22. de Wit P.J.G.M.(1986) Elicitation of active resistance mechanisms. In: "Biology and molecular biology of plantpathogen interactions". Editor J. Bailey. NATO ASI series, Volume H1. Springer Verlag. Berlin-Heidelberg, pp 149-169.

CLAIMS

1. A method for the protection of plants against pathogens,
5 wherein a polynucleotide sequence comprising at least a sequence
of a pathogen avirulence gene (E) encoding a specific elicitor
protein molecule (e) or a portion thereof is introduced into the
genome of a plant containing a corresponding resistance gene (R),
in which genes (E) and (R) are regulated in such a manner that
10 expression of said genes is only simultaneous at the site of
infection and said simultaneous expression can be induced by a
broad range of pathogens.

2. A method for the protection of plants against pathogens,
wherein a polynucleotide sequence comprising at least a sequence
15 of an avirulence gene (E) of a pathogen encoding a specific
elicitor protein molecule (e) or a portion thereof and a
corresponding resistance gene (R), are introduced into the genome
of a plant, in which genes (E) and (R) are regulated in such a
manner that expression of said genes is only simultaneous at the
20 site of infection and said simultaneous expression can be induced
by a broad range of pathogens.

3. A method according to claim 1 or 2, wherein the
simultaneous expression at the site of infection is achieved by
introducing into the genome of the plant, already containing a
25 gene (R):

- a corresponding avirulence gene (E), under the control of a
promoter (P) in which said promoter (P) can be induced by a broad
range of pathogens, and permits expression of the gene (E) only at
the site of infection.

30 4. A method according to claim 2, wherein the simultaneous
expression at the site of infection is achieved by introducing
into the genome of the plant:

- an avirulence gene (E), under the control of a promoter which
permits expression at least at the site of infection, and

35 - a corresponding resistance gene (R), under the control of a
promoter (P) in which said promoter (P) can be induced by a broad
range of pathogens, and permits expression of gene (R) only at the

site of infection.

5. A method according to any of claims 1-4, wherein the promoter P is selected from a member of the family Solanaceae.

6. A method according to claim 5, wherein the promoter is selected from a member of the genus Lycopersicon or Solanum.

7. A method according to any of claims 1-6, wherein avirulence gene (E) is derived from a plant pathogenic fungus.

8. A method according to any of claims 1-6, wherein avirulence gene (E) is derived from a plant pathogenic bacterium.

9. A method according to any of claims 1-6, wherein avirulence gene (E) is derived from a plant parasitic nematode.

10. A method according to any of claims 1-6, wherein avirulence gene (E) is derived from a plant pathogenic virus.

11. A method according to claim 7, wherein avirulence gene (E) is derived from Cladosporium fulvum.

12. A method according to claim 11, wherein the avirulence gene (E) is avr9 or a portion thereof.

13. A method according to claim 12, wherein avirulence gene avr9 comprises the nucleotide sequence as represented in figure 4.

14. A method according to any of claims 1-13, wherein resistance gene (R) is naturally present in a plant that is a member of the family of the Solanaceae.

15. A method according to claim 14, wherein resistance gene (R) is naturally present in a plant of the species Lycopersicon esculentum.

16. A method according to claim 15, wherein the resistance gene (R) is Cf9.

17. A polynucleotide sequence comprising at least a sequence of an avirulence gene (E) from a plant pathogen encoding a specific elicitor protein molecule (e) or a portion thereof, and a plant promoter (P) that can be induced by a broad range of pathogens and which permits expression at the site of infection only.

18. A polynucleotide sequence comprising at least a sequence of a resistance gene (R) and a plant promoter (P) that can be induced by a broad range of pathogens and which permits expression at the

site of infection only.

19. A plant obtained using a method according to any of the claims 1-16.

20. A plant comprising a polynucleotide sequence according
5 to claim 17 or 18.

fig-1

Oligonucleotide probes with corresponding aminoacidsequence

| | | | | | | | | | | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 |
| Tyr | Cys | Asn | Ser | Ser | Cys | Thr | Arg | Ala | Phe | Asp | Cys | Leu | Gly | Gly | Cys | Gly | Arg | Cys | Asp | Phe | His | Lys | Leu | Gln | Cys | Val |
| UAU | UCU | AAU | UCU | UCU | UCU | ACU | CCU | CCU | UUU | GAU | UCU | CCU | GGU | CAA | UCU | CCU | CCU | UCU | GAU | UUU | CAU | AAA | CUU | CAA | UCU | CUU |
| C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C |
| A | A | A | A | A | A | A | A | A | A | A | A | A | A | A | A | A | A | A | A | A | A | A | A | A | A | A |
| G | G | G | G | G | G | G | G | G | G | G | G | G | G | G | G | G | G | G | G | G | G | G | G | G | G | |
| AGU | AGU | | | | | | AGA | | | | UUA | | | | | ACA | | | | | | UUA | | | | |
| C | C | | | | | | G | | | | G | | | | | G | | | | | | G | | | G | |

PROBE A: 5' CC ACA TTG ICC IAG ACA ATC AAA 3' (a.z. 10-17)
 G C A G G C

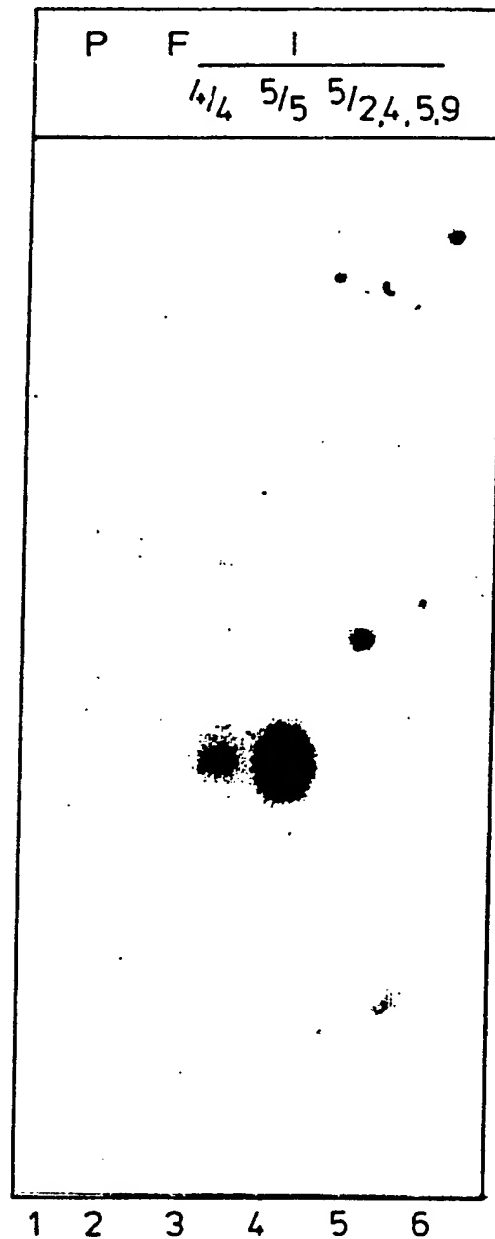
PROBE B: 5' AG TTT ATG AAA ATC ACA 3' (a.z. 19-24)
 A C G G G G

PROBE C: 5' AC ICA ITG IAG TTT ATG AAA ATC ACA 3' (a.z. 19-27)
 A C G G G G

PROBE D: 5' AC ICA ITC IAI ITT ITG IAA ITC ICA 3' (a.z. 19-27)

2/4

fig - 2

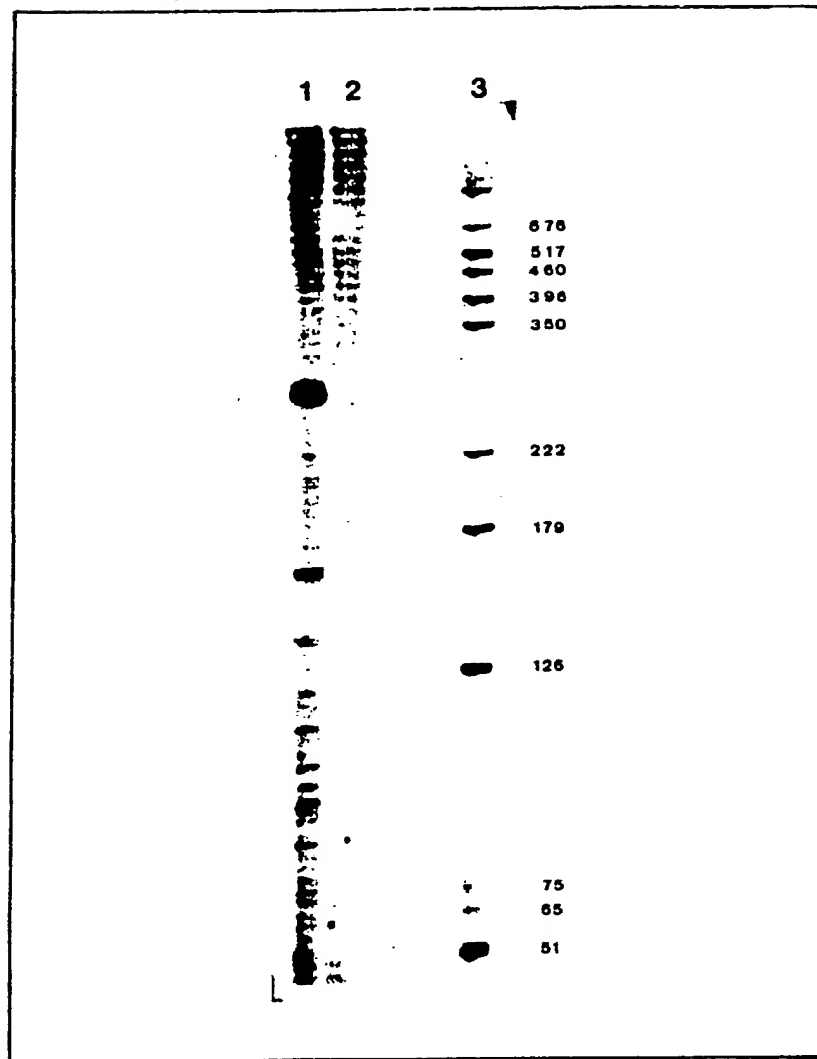
Oligonucleotide hybridisation

- 1 = Non infected tomato
- 2 = In vitro cultured C fulvum
- 3 = Cultivar Cf4 / race 4
- 4 = Cultivar Cf5 / race 5
- 5 = Cultivar Cf5 / race 2.4.5.9.11

3/4

fig - 3

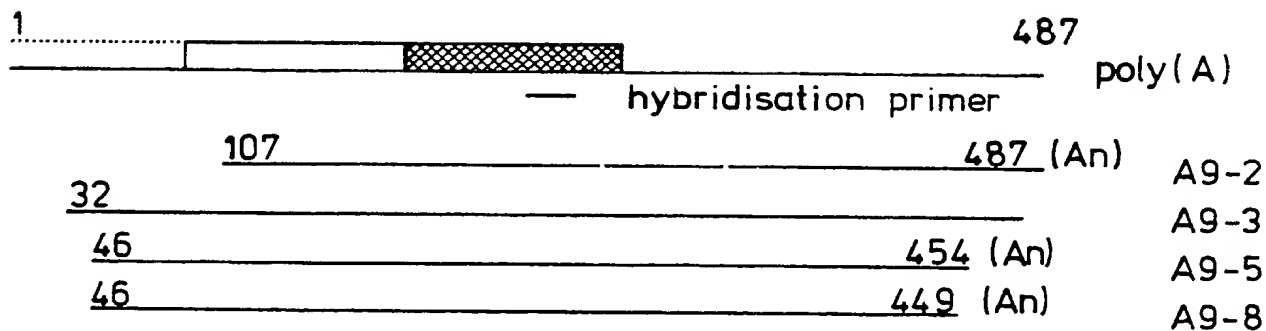
Poly ARNA extension products



1 = Poly ARNA of Cf 5/ race 5
2 = Poly ARNA of Cf 5/ race 2.4.5.9.11

fig - 4

Sequence of avr9 mRNA and the structure of the corresponding
cDNA clones



— RNA sequence primer

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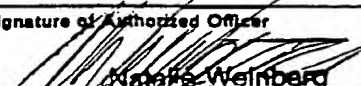
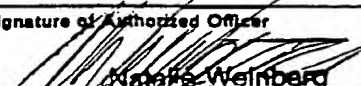
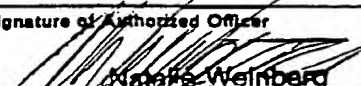
1  AACACTAGCAATTTGSAATTCCTCAAGCCTACTAAGGTCCTTTGTAGCCTAATGTCATTT  60
      M K L S L L S V E L A
61  TAATAAGTCTATGACTTAAGTATCTATCATGAGCTTTTCCCTCCTTAGCGTAGAGCTTG  120
      . . . . .
      L L I A T T L P L C W A A A L P V G L G
121 CTCTCCTAATTGCTACTACTCTCCTCCTTTGCTGGGCGAGCTGCGCTCCTCTGTAGGATTTG  180
      . . . . .
      V G L D Y C N S S C T R A F D C L G Q C
181 GAGTGGGCTAGACTACTGTAACTCAAGTTGTACTAGGGCTTGGACTGTTTGGGTCGAT  240
      . . . . .
      G R C D F H K L G C V H *
241 GTGGCAGATGCGACTTTTCATAAGCTACAATGTGTGCTCACTAGAGGACTAGAGAGGAGTGG  300
      . . . . .
301 AGAGAAGAGGAGGGGAGAGGTAAGATACTAGCGAGTAATCGTACAGGTAGAAAGGGAT  360
      . . . . .
361 AGTAAGCAGGAGATAGAGGAGGAGCTTGGGACCTTATCCACATAAGTCTAGTCTGTA  420
      . . . . .
421 ACATTTGTTTATATTGAAGGCTTTTCTCCTAATAGTTTCTCAATGTGCTGCGAGGCGCAG  480
      . . . . .
481 AGGCAAG  487

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/NL 91/00052

| I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁵ : C 12 N 15/31, C 12 N 15/82, A 01 N 63/02 | | | | | | | | | | | | | | |
|--|--|-------------------------------------|--|--|--|---|--|------|---|--|------|-----|--|--|
| II. FIELDS SEARCHED <div style="text-align: center; font-size: small;">Minimum Documentation Searched ⁷</div> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 25%; padding: 5px;">Classification System</td> <td style="padding: 5px;">Classification Symbols</td> </tr> <tr> <td style="padding: 5px;">IPC⁵</td> <td style="padding: 5px;">C 12 N, A 01 N</td> </tr> </table> <div style="text-align: center; font-size: x-small;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div> | | | Classification System | Classification Symbols | IPC ⁵ | C 12 N, A 01 N | | | | | | | | |
| Classification System | Classification Symbols | | | | | | | | | | | | | |
| IPC ⁵ | C 12 N, A 01 N | | | | | | | | | | | | | |
| III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; padding: 5px;">Category ⁹</th> <th style="width: 70%; padding: 5px;">Citation of Document, ¹¹ with Indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%; padding: 5px;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">WO, A, 8605516 (DUKE UNIVERSITY) 25 September 1986 see page 32, lines 2-13 --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-20</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">EP, A, 0337532 (MOGEN) 18 October 1989 see column 6, lines 23-42 --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-20</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">P,X</td> <td style="padding: 5px;">Biological Abstracts, volume 91, 1991, J.A.L. Van Kan et al.: "Cloning and characterization of complementary DNA of avirulence gene avr9 of the fungal pathogen Cladosporium fulvum, causal agent of tomato leaf mold", page 499, abstract no. 72989 & Mol Plant-Microbe Interact 4(1): 52- 59, 1991 -- ./. </td> <td></td> </tr> </table> | | | Category ⁹ | Citation of Document, ¹¹ with Indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ | Y | WO, A, 8605516 (DUKE UNIVERSITY) 25 September 1986 see page 32, lines 2-13 -- | 1-20 | Y | EP, A, 0337532 (MOGEN) 18 October 1989 see column 6, lines 23-42 -- | 1-20 | P,X | Biological Abstracts, volume 91, 1991, J.A.L. Van Kan et al.: "Cloning and characterization of complementary DNA of avirulence gene avr9 of the fungal pathogen Cladosporium fulvum, causal agent of tomato leaf mold", page 499, abstract no. 72989 & Mol Plant-Microbe Interact 4(1): 52- 59, 1991 -- ./. | |
| Category ⁹ | Citation of Document, ¹¹ with Indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ | | | | | | | | | | | | |
| Y | WO, A, 8605516 (DUKE UNIVERSITY) 25 September 1986 see page 32, lines 2-13 -- | 1-20 | | | | | | | | | | | | |
| Y | EP, A, 0337532 (MOGEN) 18 October 1989 see column 6, lines 23-42 -- | 1-20 | | | | | | | | | | | | |
| P,X | Biological Abstracts, volume 91, 1991, J.A.L. Van Kan et al.: "Cloning and characterization of complementary DNA of avirulence gene avr9 of the fungal pathogen Cladosporium fulvum, causal agent of tomato leaf mold", page 499, abstract no. 72989 & Mol Plant-Microbe Interact 4(1): 52- 59, 1991 -- ./. | | | | | | | | | | | | | |
| <div style="font-size: x-small;"> ⁹ Special categories of cited documents: ¹⁰ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family </div> | | | | | | | | | | | | | | |
| IV. CERTIFICATION <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; padding: 5px;"> Date of the Actual Completion of the International Search <div style="text-align: center;">12th July 1991</div> </td> <td style="width: 50%; padding: 5px;"> Date of Mailing of this International Search Report <div style="text-align: center;">13. 08. 91</div> </td> </tr> <tr> <td style="padding: 5px;"> International Searching Authority <div style="text-align: center;">EUROPEAN PATENT OFFICE</div> </td> <td style="padding: 5px;"> Signature of Authorized Officer <div style="text-align: center;">  Nikola Weinberg </div> </td> </tr> </table> | | | Date of the Actual Completion of the International Search <div style="text-align: center;">12th July 1991</div> | Date of Mailing of this International Search Report <div style="text-align: center;">13. 08. 91</div> | International Searching Authority <div style="text-align: center;">EUROPEAN PATENT OFFICE</div> | Signature of Authorized Officer <div style="text-align: center;">  Nikola Weinberg </div> | | | | | | | | |
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| International Searching Authority <div style="text-align: center;">EUROPEAN PATENT OFFICE</div> | Signature of Authorized Officer <div style="text-align: center;">  Nikola Weinberg </div> | | | | | | | | | | | | | |

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) | | |
|--|---|-----------------------|
| Category * | Citation of Document, ** with indication, where appropriate, of the relevant passages | Relevant to Claim No. |
| A | Trends in Genetics, volume 5, no. 2, February 1989, Elsevier Science Publishers Ltd, (GB), D. Baulcombe: "Strategies for virus resistance in plants", pages 56-60 see page 59, right-hand column - page 60, left-hand column | 1-20 |
| | -- | |
| A | Physiological and Molecular Plant Pathology, volume 33, no. 1, 1988, Academic Press, Ltd., I.M.J. Schottens-Toma et al.: "Purification and primary structure of a necrosis- inducing peptide from the apoplastic fluids of tomato infected with Clados- porium fulvum (syn. Fulvia fulva)", pages 59-67 see the whole document | 7,11-13,14- 16 |
| | -- | |
| A | Biological Abstracts, volume BR39, M.J. Dickinson et al.: "Strategies for cloning gene for resistance to fulvia- fulva from tomato", see abstract no. 65954 & J. Exp Bot. 41 (Suppl.) 1990, p6-3 | 18,20 |
| | -- | |
| A | Molecular Genetics of Plants-Microbe Interactions, 15-20 May 1988, APS Press, (St. Paul, Minnesota, US), R.P. Oliver et al.: "Molecular approa- ches for the study of the pathogenici- ty of fulvia-fulva", pages 263-265 see the whole document | 7,11-16 |
| | -- | |
| A | Foundation for Biotechnical and Industrial Fermentation Research, volume 6, 2-7 July 1989, (Espoo, FI), F.J.G.M. De Wit et al.: "The interaction between Cladosporium-fulvum synonym fulvia-fulva and tomato a model system in molecular plant pathology", pages 227-236 see the whole document | 7,11-16 |
| | -- | |
| A | Biofutur, January 1989, M.H. Lebrun: "La biologie moléculaire s'attaque aux maladies des plantes", pages 56-58 see the whole document | 1,7-10 |
| | -- | |

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, " with indication, where appropriate, of the relevant passages | Relevant to Claim No. |
|------------|---|-----------------------|
| A | EP, A, 0298918 (CIBA-GEIGY) 11 January 1989 see the whole document ----- | 1,7-10 |

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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

NL 9100052

SA 46424

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The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|---------------------------------|----------------------|
| WO-A- 8605516 | 25-09-86 | EP-A- 0215907 JP-T- 62502863 | 01-04-87 19-11-87 |
| EP-A- 0337532 | 18-10-89 | NL-A- 8800725 JP-A- 2042991 | 16-10-89 13-02-90 |
| EP-A- 0298918 | 11-01-89 | JP-A- 1037294 | 07-02-89 |

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